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World J Gastroentero, 2001; 7(1):102 - 106
World Journal of Gastroenterology
 ISSN 1007 - 9327

Humoral and cellular immunogenecity of DNA vaccine based on hepatitis B core gene in rhesus monkeys

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Subject headings hepatitis B virus; DNA vaccine; hepatitis B core antigens; nucleic acid vaccine; enzyme-linked immunosorbent assay; macaca mulatta

Huang ZH, Zhuang H, Lu S, Guo RH, Xu GM, Cai J, Zhu WF. Humoral and cellular immunogenecity of DNA vaccine based on hepatitis B core gene in rhesus monkeys. ***World J Gastroenterol***, 2001;7(1):102-106

INTRODUCTION

Hepatitis B virus (HBV) is the most common etiologic agent for infectious liver diseases. It is estimated that there are more than 250 million chronic HBV carriers in the world today and there is a significant association among persistent infection, liver cirrhosis and hepatocellular carcinoma[1-3]. The control of HBV infections is thought to be mediated by both humoral and cellular immune responses involving neutralizing antibodies as well as class I and class II major histocompatibility complex (MHC)-restricted T- cells[4,5]. Among the HBV antigens, a number of studies have highlighted the importance of the human immune response against the HBcAg and HBeAg during HBV infections. During acute HBV infection, cytotoxicity T lymphocyte (CTL) specific for HBcAg and HBeAg can be detected in the circulation of the infected host. In contrast, in chronic HBV infection, HBcAg and HBeAg-specific CTL and T-helper cell activity are not readily detected. The cumulated data suggest that CTL activity may play an important role in resolving HBV infection[6-11].

DNA mediated immunization has been shown to be an novel method to induce both humoral and cell-mediated immune responses against many different antigens including HBV antigens[12,13]. We have demonstrated that the DNA vaccine based on HBV core gene has strong humoral and cellular immunogenecity in different species of mice[14,15]. In our experiments, we have further investigated the immunogenecity of this DNA vaccine in rhesus monkeys. The results show that the DNA vaccine of HBV core gene can prime obvious antigen-specific antibody and cell mediated immune responses.

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Project supported by the grant from Science Foundation of Ministry of Health of China, No.96-1-347.
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Received 2000-08-08 **Accepted** 2000-10-29

MATERIALS AND METHODS

Preparation of DNA vaccine of HBV core gene

The control plasmid (pJW4303) and DNA vaccine of HBV core gene (pJW4303/HBc) were propagated by a large amount of culture of the transformed *E.coli* strain of HB101. Plasmid DNA was purified with QIAGEN Plasmid Mega Kit (QIAGEN, Germany).

Rhesus monkeys

Four rhesus monkeys (2 male, 2 female, 3 years of age) were purchased from Special Animal Breeding and Raising Center, Xingye, Henan Province, China and maintained at the animal house in Beijing Medical University. The monkeys were divided into experimental group and control group (2 monkeys in each group).

Protocols of DNA immunization

The monkeys in the experimental group were immunized with plasmid pJW4303/HBc and that in the control group were immunized with plasmid pJW4303. The plasmids were dissolved in normal saline to a final concentration of 1 g/L. Each time one monkey received 4-site intramuscular injections with a total volume of 2 mL plasmid solution containing 2 mg plasmid DNA. Three boosts with same dose were given at an interval of 2 months. The monkeys' sera before and after immunizations were collected and stored at -30℃.

Detection of anti-HBc antibody

Anti-HBc antibodies in monkeys' sera were first detected by Abbott Imx System (Abbott, USA) according to the manufacturer's instructions and end-point titers of anti-HBc antibody were then detected by an enzyme linked immunosorbent assay (ELISA). The procedures were as follows: ① The 96-well microplates were coated with recombinant HBcAg (1 mg/L) and blocked with PBS containing

10% FCS. ② Three-fold dilutions of monkeys' sera (1: 50, 1:150, 1:450 …… 1:984150) were added to triplicate wells. ③ HRP labeled rabbit anti-human IgG (Sino-American Biotechnology Co.) at the dilution of 1:3000 was used as second antibody. ④ The substrate solution (TMB) was then added to each well and reaction was stopped by 2M H_2SO_4. ⑤ The absorbence value was measured at wavelength of 450 nm by an ELISA reader. Microplate washings were performed between each step with PBST solution. The end-point of anti-HBc titer was defined as the highest serum dilution that resulted in an absorbence value two times that of non-immune or control serum.

Detection of IgG subclasses of anti-HBc

Subclasses of anti-HBc antibodies were detected in the sera of the monkeys positive for anti-HBc. The procedures were similar to the ELISA method mentioned above for detecting anti-HBc, except that serum was diluted to 1:30. 1:500 diluted sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Nordic Immunological Laboratories, Tilburg, the Netherlands) were used as the second antibody, and 1:5000 diluted HRP labeled rabbit anti-sheep IgG (Jackson Immuno-Research Laboratories Inc, PA, USA) was used as the third antibody.

Detection of IFN-γ and IL-4 in PBMC culture supernatant

The procedures were as follows: ① PBMCs were separated from heparinized monkey blood by Ficoll gradient sedimentation method. ② PBMCs were resuspended with RPMI-1640 containing 10% FCS to a final concentration of 2×10^6 cells/ mL. ③ PBMC suspension 250 μL (5×10^5 cells) was added to triplicate wells in a 24-well cell culture plate, and recombinant human IL-2 (500U/well) was added as well. ④ Except for control wells, PBMCs in each triplicate wells were restimulated with recombinant HBcAg at different doses of 5 μg/well, 10 μg/well and 12.5 μg/well. ⑤ After 48 h incubation under the condition of 37℃, 5% CO_2, the supernatant was collected from each well and stored at once at -70℃. ⑥ IFN-γ and IL-4 concentrations were detected by the ELISA kits (Jinmei Biotechnology Co., Shenzheng, China).

PBMC proliferation assay

The procedures were similar to that for detecting IFN-γ and IL-4 in PBMC culture supernatant, except that ① PBMCs were incubated for 72 h; ② 0.5 μCi ^{3}H-TdR was added to each well and followed by another 4h incubation; PBMCs were then collected onto filter membrane which were then backed 2 h at 80℃; and the radioactivity (CPM) was determined by a beta-scintillation counter (Beckman). The PBMC proliferation activity was expressed by Stimulation Index (SI), which was calculated according to the following formula: (SI = CPM of HBcAg stimulated well/CPM of non HBcAg stimulated well). SI value greater than 2 was generally considered as having antigen specific PBMC proliferation.

RESULTS

Anti-HBc IgG and its end-point titer in monkey's sera

The results of anti-HBc IgG and its end-point titer in monkey's sera are shown in Table 1.

Table 1 Anti-HBc in sera of experimental and control monkeys

Monkey No.	Group	Anti-HBc antibody					End-point
		0 month	2 month	4 month	6 month	8 month	
1	Experimental	N[a]	P[b]	N/D[c]	N/D	N/D	1:36450
2	Experimental	N	N	P	P	P	1:109350
3	Control	N	N	N	N	N	1:150
4	Control	N	N	N	N	N	1:150

[a] N: negative

[b] P: positive (in Abbott Imx System, the detected value less than 1. 00 was considered positive for anti-HBc).

[c] not detected because of death.

Subclasses of anti-HBc IgG in sera of experimental group of rhesus monkeys

Subclasses of anti-HBc IgG (IgG1, IgG2, IgG3 and IgG4) were detected in the experimental monkeys (No.1 and No.2), which were found to be positive for anti-HBc in the previous tests. The results are shown in Table 2.

Table 2 Subclasses of anti-HBc IgG and IgG1/IgG2 ratio in rhesus monkeys' sera

Monkey No.	IgG1	IgG2	IgG3	IgG4	IgG1/IgG2
1	0.61 + 0.01[a]	1.02 + 0.08	0.32 + 0.02	0.12 + 0.01	0.60
2	0.61 + 0.04	1.05 + 0.04	0.40 + 0.01	0.18 + 0.03	0.58

[a] The values indicated $\bar{x} \pm s$ of triplicate wells.

IFN-γ and IL-4 levels in culture supernatant of PBMCs stimulated with recombinant HBcAg

IFN-γ and IL-4 levels were detected in monkey No.2 (experimental group) and monkey No.3 (control group). Monkey No.1 and No.4 died before this test was performed. The results are shown in Table 3.

Table 3 IFN-γ and IL-4 values in culture supernatant of PBMCs

Monkey No.	Group	IFN-γ (ng/L)	IL-4 (ng/L)
2	Experimental	15.63	6.25
3	Control	<3.13	6.25

HBcAg specific PBMCs proliferation activity in experimental and control groups of rhesus monkeys

HBcAg specific PBMCs proliferation activities were measured in monkey No.2 and No.3 by the time of 12 months after first immunization. The results are listed in Table 4.

Table 4 HBcAg specific PBMC proliferation activity*

Monkey No.	Group	HBcAg dose for stimulation (μg/well)			
		0	5	10	12.5
2	Experimental	354.4 ± 64.5	984.9 ± 105.4[a] (2.74)	1364.9 ± 47.9[a] (3.83)	890 ± 155.6[a] (2.12)
3	Control	198.4 ± 3.9	274.5 ± 33.2 (1.37)	261.5 ± 28.2 (1.32)	250 ± 70.0 (1.24)

*The values in the table refer to CPM ($x \pm s$ from each triplicate well), the values in the brackets indicate stimulation index (SI). [a]$P<0.05$ *vs* control monkey.

DISCUSSION

DNA-mediated immunization refers to the induction of an immune response to antigen expressed *in vivo* subsequent to the introduction of DNA carrying the protein coding sequences and the regulatory elements needed to express them[16,17]. An important feature of DNA-based immunization is the *in situ* production of the expressed protein (s), mimicking a viral infection. The endogenous synthesis should allow presentation of antigens by class I molecules of MHC, resulting in the induction of CD8 + cytotoxic T lymphocytes (CTL)[18]. There have been several experimented reports in which recombinant plasmid DNA was used to induce immune responses to particular pathogens, including malaria[19], herpes simplex virus (HSV)[20], influenza A[21], rabies virus[22], simian immunodeficiency virus (SIV)[23], human immunodeficiency virus type I (HIV)[24] and hepatitis B virus (HBV)[25-32].

In our earlier work, the HBV core gene fragment, which was modified to assure the high level expression of HBcAg[33], was successfully cloned into the plasmid pJW4303, the vector containing CMV immediate early promotor. This recombinant plasmid was named pJW4303/HBc. The DNA immunization using pJW4303/HBc among Balb/c (H_2d) and C57BL/6 (H_2b) mice showed that this recombinant could induce strong humoral (antibody) and cellular (CTL) immune responses[34].

When evaluating the immunogenecity and safety of potential DNA vaccine for eventual use in humans, the nonhuman primate models should be considered. The best nonhuman primate candidate would be those closest to humans on a phylogenetic basis. However, cost and other considerations may preclude studies in hominoid species, such as chimpanzee, orang utans, gorillas, and gibbons. Based on the cost and availability, nonhominoid primate species including rhesus monkeys, represent the alternative candidate nonhuman primate species for pre-clinical immunogenecity studies[35,36].

Townsend *et al*[37] observed the specific immune responses in mouse and rhesus monkeys after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. Their results showed that intramuscular injections with 10^8 CFU of the the LHBc-Neo retrovirus vector into rhesus monkeys induced HBc/eAg-specific antibody production and CD8 + CTLs. The CTL response is long-lasting, and being detectable as late as 16 weeks after immunization.

We used the plasmid as the vector to carry HBV core gene for DNA immunization in rhesus monkeys, which was different from the observation above the reason for that is that the safety of the vector for retrovirus vector was integratable to the host genome.

In our experiments, all 4 monkeys were negative for anti-HBc before DNA immunization. After intramuscular immunization of pJW4303/HBc and pJW4303, the monkeys in the experimental group all developed anti-HBc antibody while the monkeys in the control group all negative for this antibody, indicating that this DNA vaccine could induce antigen specific humoral response in rhesus monkeys. We also found that the monkeys in the experimental group could show different antibody response profiles. Monkey No.1 became positive for anti-HBc (1:36450) after the first immunization while monkey No.2 was not negative for anti-HBc until the second immunization and the antibody titer became higher (1: 103 950) as late as the total four immunizations were accomplished. This different antibody production profiles might indicate the individual difference in response to DNA immunization.

In human and other hominoid primates, the serum IgG exhibited four subclasses, i.e., IgG1, IgG2, IgG3 and IgG4. The relative concentrations of these IgG subclasses were 60%-70% for IgG1, 15%-20% for IgG2, 5%-10% for IgG3, and 1%-7% for IgG4. When looked into the antigen specific IgG antibodies the concentration of IgG1 and IgG2 and its ratio IgG1/IgG2 could reflect the response profiles of helper T cells (T-H1 type or T-H2 type) to some extent. Generally speaking, IgG1/IgG2<1 or IgG1/IgG2>1 reflected T-H1 type or T-H2 type immune responses. The previous data showed that T-H1 type response was beneficial for the clearance or eradication of chronic infected viruses while the T-H2 type- response was usually correlated to the exacerbation of immunopathogenic damage of host tissues[38]. Feltquate *et al* had found that intramuscular immunization of DNA vaccines was prone to induce T-H1 type of immune response, thereby facilitating the recovery of the host from chronic viral infection[39]. Our results also

demonstrated that two monkeys intramuscularly immunized with HBV core DNA vaccine all exhibited T-H1 type of immune response based on the fact that their IgG1/IgG2 ratios were all less than 1 (0.60 and 0.58, respectively).

The profiles of cytokine production were another indicators of helper T cell responses[40-44]. IFN-γ, IL-2, TNF-α and GM-CSF were usually considered as T-H1 type cytokines, while IL-4, IL-5 and IL-10 were T-H2 type cytokines. IFN-γ and IL-4 were chosen in this experiment to observe helper T cell responses after DNA immunization of HBV core gene in rhesus monkeys. IFN-γ level was significantly higher in the culture supernatant of PBMC from the monkeys immunized with HBV core DNA vaccine than that from monkeys injected only with control plasmid (15.63 ng/L *vs* <3.13 ng/L). At the same time, IL-4 levels in both monkeys with injections of pJW4303/HBc or PJW4303 were similar (6.25 ng/L *vs* 6.25 ng/L). The results indicated IFN-γ prominent cytokine profile in the monkey immunized with HBV core DNA vaccine. This result combined with the result of IgG1/IgG2 ratios mentioned above further confirmed the T-H1 type immune responses in the monkeys of the experimental group.

Cell-mediated immune response is critical for the termination of chronic HBV infections[45-47]. Antigen specific lymphocyte proliferation assay is an alternative for the CTL assay to evaluate the cell-mediated immune response[48]. In this experiment, the HBcAg specific PBMC proliferation activity was seen in the monkey immunized with pJW4303/HBc but not in the monkey injected with pJW4303 ($P<0.05$). After stimulation with three different doses of HBcAg, the stimulation index (SI) was all >2 in the experimental monkeys but all <2 in the control monkeys, which strongly indicated that DNA vaccine of pJW4303/HBc could induce antigen-specific cell-mediated immune response in rhesus monkeys.

Sallberg *et al* reported that DNA immunization of HBV core gene using retrovirus as vector could markedly decrease the HBV DNA level in the sera of experimental chimpanzees, and even induce the seroconversion of HBeAg to anti-HBe[49]. Our results showed that using plasmid as vector the DNA vaccine could also stimulate the immune responses in nonhuman primate rhesus monkeys, which was obviously helpful and beneficial for the host to inhibit and eventually eradicate chronically infected virus, including hepatitis B virus. As the designer vaccines for the 21st century, DNA vaccines demonstrated its feasibility of inducing specific cellular immunity in humans[50]. We believed that DNA vaccine of HBV core gene may become a potential therapeutics for the treatment of chronic HBV infection in humans in the near future.

REFERENCES

1 Chan HL, Ghany MG, Lok ASF. Hepatitis B. Schiff's diseases of the liver. 8th Ed. Philadelphia: Lippincott-Raven Publishers, 1999: 757-784
2 Gerlich WH, Thomssen R.The viruses of hepatitis. In: 2nd ed. Bircher J, Benhamou JP, McIntyre N, eds. Oxford textbook of clinical hepatology. Oxford: *Oxford University Press*, 1999: 828-954
3 Wang XL, Guo H, Li ZZ, Jia SY, Zhang ZR, Liu H. Hemodynamic study of HBV carriers. *World J Gastroenterol*, 1998;4(Suppl 2):85
4 Milich DR. Immune response to the hepatitis B virus: infection, animal models, vaccination. *Viral Hepat Rev*, 1997;3:63-103
5 Bertoletti A, Southwood S, Chesnut R, Sette A, Falco M, Ferrara GB, Penna A, Boni C, Fiaccadori F, Ferrari C. Molecular features of the hepatitis B virus nucleocapsid T-cell epitope 18-27: interaction with HLA and T-cell receptor. *Hepatology*, 1997;26:1027-1034
6 Lee WM. Hepatitis B virus infection. *N Engl J Med*, 1997;337: 1733-1745
7 Ferrari C, Penna A, Bertoletti A, Valli A, Antoni AD, Giuberti T, Cavalli A, Petit MA, Fiaccadori F. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J Immunol*, 1990;145:3442-3449
8 Bertoletti A, Ferrari C, Fiaccadori F, Penna A, Margolskee R, Schlicht HJ, Fowler P, Guilhot S, Chisari FV. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. *Proc Natl Acad Sci USA*, 1991;88:10445-10449
9 Penna A,Chisari FV,Bertoletti A, Missale G, Fowler P, Giuberti T, Fiaccadori F, Ferrari C. Cytotoxic T lymphocytes recognize an HLA-restricted epitope within the hepatitis B virus nucleocapsid antigen. *J Exp Med*, 1991;174:1565-1570
10 Tsai SL, Chen PJ, Lai MY, Yang PM, Sung JL, Huang JH, Hwang LH, Chang TH, Chen DS. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. *J Clin Invest*, 1992;89:87-96
11 Lau GKK. Immunological approaches to the breakdown of hepatitis B viral persistence. *World J Gastroenterol*, 1998;4(Suppl 2): 32
12 Robinson HL. Nucleic acid vaccines: an overview. *Vaccine*, 1997; 15:785-787
13 Zhao LS, Qin S, Zhou TY, Tang H, Liu L, Lei BJ. DNA-based vaccination induces humoral and cellular immune responses against hepatitis B virus surface antigen in mice without activation of C-myc. *World J Gastroenterol*, 2000;6:239-243
14 Huang ZH, Lu S, Liu N, Wang SX. Specific immune responses in H-2^d mice after DNA immunization of HBV core gene. *ZhonghuaGanzangbing Zazhi*, 1999;7:107-109
15 Huang ZH, Lu S, Liu N, Wang SX. Specific cellular and humoral immune responses in H-2^b mice induced by DNA immunization of HBV core gene. *Zhonghua Chuanranbing Zazhi*, 1999;17:104-107
16 Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle *in vivo*. *Science*, 1990;247:1465-1468
17 Tang DC, Devit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature*, 1992;356:152-154
18 McDonnell WM, Askari FK. Molecular medicine DNA vaccines. *N Engl J Med*, 1996;334:42-45
19 Hoffman SL, Doolan DL, Sedegah M, Wang R, Scheller LF, Kumar A, Weiss WR, Le TP, Klinman DM, Hobart P, Norman JA, Hedstrom RC. Toward clinical trials of DNA vaccines against malaria. *Immunol Cell Biol*, 1997;75:376-381
20 Manickan E, Rouse RJD, Yu Z, Wire WS,Rouse BT. Genetic immunization against herpes simplex virus: protection is mediated by CD4$^+$ T lymphocytes. *J Immunol*, 1995;155:259-265
21 Justewicz DM, Morin MJ, Robinson HL, Webster RG. Antibody-forming cell response to virus challenge in mice immunized with DNA encoding the influenza virus hemagglutinin. *J Virol*, 1995; 69:7712-7717
22 Xiang ZQ, Spitalnik S, Tran M, Wunner WH,Cheng J, Ertl HCJ. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology*, 1994;199:132-140
23 Yasutomi Y, Robinson HL, Lu S, Mustafa F, Lekutis C, Arthos J, Mullins JI, Voss G, Manson K, Wyand M, Letvin NL. Simian immunodeficiency virus-specific cytotoxic T-lymphocyte induction through DNA vaccination of rhesus monkeys. *J Virol*, 1996; 70:678-681
24 Lu S, Santoro JC, Fuller DH, Haynes JR, Robinson HL. Use of DNAs expressing HIV-1 env and noninfectious HIV-1 particles to raise antibody responses in mice. *Virology*, 1995;209:147-154
25 Davis HL, McCluskie MJ, Gerin JL, Purcell RH.DNA vaccine for

hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. *Proc Natl Acad Sci USA*, 1996; 93:7213-7218
26 Davis HL, Millan CLB, Mancini M, McCluskie MJ, Hadchouel M, Comanita L, Tiollais P, Whalen RG, Michel ML. DNA-based immunization against hepatitis B surface antigen (HBsAg) in normal and HBsAg-transgenic mice. *Vaccine*, 1997;15:849-852
27 Schirmbeck R, Böhm W, Ando K, Chisari FV, Reimann J. Nucleic acid va ccination primes hepatitis B virus surface antigena-specific cytotoxic T lymphocytes in nonresponder mice. *J Virol*, 1995;69:5929-5934
28 Michel ML, Davis HL, Schleef M, Mancini M, Tiollais P, Whalen RG. DNAa-mediated immunization to the hepatitis B surface antigen in mice: Aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc Natl Acad Sci USA*, 1995;92: 5307-5311
29 Mancini M, Hadchouel M, Davis HL, Whalen RG, Tiollais P, Michel ML.DNA- mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. *Proc Natl Acad Sci USA*, 1996;93:12496-12501
30 Sllberg M, Townsend K, Chen M, O dea J, Banks T, Jolly DJ, Chang SM, Lee WT, Milich DR. Characterization of humoral and $CD4^+$ cellular responses after genetic immunization with retroviral vectors expressing different forms of the hepatitis B virus core and e antigens. *J Virol*, 1997;71:5295-5303
31 Kuhröber A, Wild J, Pudollek HP, Chisari FV, Reimann J. DNA vaccination with plasmids encoding the intracellular (HBcAg) or secreted (HBeAg) form of the core protein of hepatitis B virus primes T cell responses to two overlapping Kaba- and Kada-restricted epitopes. *Inter Immunol*, 1997;9:1203-1212
32 Geissler M, Tokushige K, Chante CC, Zurawski VR Jr, Wands JR. Cellular and humoral immune response to hepatitis B virus structural proteins in mice after DNA-based immunization. *Gastroenterology*, 1997;112:1307-1320
33 Ye YA, Zhan MY. High efficient expression in E.coli of hepatitis B virus core gene after 5 end modification. *Bingdu Xuebao*, 1988; 4:312-318
34 Huang ZH, Lu S, Liu N, Wang SX, Li J, Tang BY.Humoral and cellular immunogenecity of genetic vaccine on core gene of hepatitis B virus in mice. *Zhonghua Yixue Zazhi*, 1999;79:456-459
35 Kennedy RC, Shearer MH, Hildebrand W.Nonhuman primate models to evaluate vaccine safety and immunogenicity. *Vaccine*, 1997; 15:903-908
36 Liu MA, McClements W, Ulmer JB, Shiver J, Donnelly J. Immunization of non-human primates with DNA vaccines. *Vaccine*, 1997;15:909-912
37 Townsend K, Sällberg M, O dea, J, Banks T, Driver D, Sauter S, Chang SM, Jolly DJ, Mento SJ, Milich DR, Lee WTL. Characterization of $CD8^+$ cytotoxic Ta-Lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. *J Virol*, 1997;71:3365-3374
38 Scott P, Kaufmann SHE. The role of T-cell subsets and cytokines in the regulation of infection. *Immunol Today*, 1991;12:346-348
39 Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T. Helpercell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol*, 1997;158:2278-2284
40 Actor JK, Shirai M, Kullberg MC, Buller R ML, Sher A, Berzofsky JA. Helminth infection results in decreased virus-specific $CD8^+$ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. *Proc Natl Acad Sci USA*, 1993;90:948-952
41 Clerici M, Hakim FT, Venzon DJ, Blatt S, Hendrix CW, Wynn TA, Shearer GM. Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J Clin Invest*, 1993;91:759-765
42 Milich DR, Wolf SF, Hughes JL, Jones JE. Interleukin 12 suppresses autoantibody production by reversing helper T-cell phenotype in hepatitis B e antigen transgenic mice. *Proc Natl Acad Sci USA*, 1995;92:6847-6851
43 Milich DR, Peterson DL, Sch¡éidel F, Jones JE, Hughes JL. Preferential recognition of hepatitis B nucleocapsid antigens by Th1 or Th2 cells is epitopeand major histocompatibility complex dependent. *J Virol*, 1995;69:2776-2785
44 Geissler M, Gesien A, Tokushige K, Wands JR. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J Immunol*, 1997;158:1231-1237
45 Lee HG, Lim JS, Lee KY, Choi YK, Choe IS, Chung TW, Kim K. Peptide-Specific CTL induction in HBV-seropositive PBMC by stimulation with peptides *in vitro*: novel epitopes identified from chronic carriers. *Virus Res*, 1997;50:185-194
46 Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, Eichenlaub D, Frosner G, Will H, Pape GR. Activation of a heterogeneous hepatitis B core and e antigen-specific $CD4^+$ T-cell population during seroconversion to antia-HBe and anti-HBs in hepatitis B virus infection. *J Virol*, 1995;69:3358-3368
47 Mondelli M, Vergani GM, Alberti A, Vergani D, Portmann B, Eddleston ALWF, Williams R. Specificity of T lymphocyte cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infection: evidence that T cells are directed against HBV core antigen expressed on hepatocytes. *J Immunol*, 1982;129:2773-2778
48 Löhr HF, Weber W, Schlaak J, Goergen B, Büschenfelde KHMZ, Gerken G. Proliferative response of $CD4^+$ T cells and hepatitis B virus clearance in chronic hepatitis with or without hepatitis B eminus hepatitis B virus mutants. *Hepatology*, 1995;22:61-68
49 Sällberg M, Hughes J, Javadian A, Ronlov G, Hultgren C, Townsend K, Anderson CG, O Dea J, Alfonso J, Eason R, Murthy KK, Jolly DJ, Chang SM, Mento SJ, Milich D, Lee WT. Genetic immunization of chimpanzees chronically infected with the hepatitis B virus, using a recombinant retroviral vector encoding the hepatitis B virus core antigen. *Hum Gene Ther*, 1998;9:1719-1729
50 Seder RA, Gurunathan S. DNA vaccines - designer vaccines for the 21st century. *N Engl J Med*, 1999;341:277-278

Edited by Ma JY